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Review

Allosteric modulation of metabotropic glutamate receptors: Structural insights and therapeutic potential

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ABSTRACT

Allosteric modulation of G protein-coupled receptors (GPCRs) represents a novel approach to the development of probes and therapeutics that is expected to enable subtype-specific regulation of central nervous system target receptors. The metabotropic glutamate receptors (mGlus) are class C GPCRs that play important neuromodulatory roles throughout the brain, as such they are attractive targets for therapeutic intervention for a number of psychiatric and neurological disorders including anxiety, depression, Fragile X Syndrome, Parkinson's disease and schizophrenia. Over the last fifteen years, selective allosteric modulators have been identified for many members of the mGlu family. The vast majority of these allosteric modulators are thought to bind within the transmembrane-spanning domains of the receptors to enhance or inhibit functional responses. A combination of mutagenesis-based studies and pharmacological approaches are beginning to provide a better understanding of mGlu allosteric sites. Collectively, when mapped onto a homology model of the different mGlu subtypes based on the β_2-adrenergic receptor, the previous mutagenesis studies suggest commonalities in the location of allosteric sites across different members of the mGlu family. In addition, there is evidence for multiple allosteric binding pockets within the transmembrane region that can interact to modulate one another. In the absence of a class C GPCR crystal structure, this approach has shown promise with respect to the interpretation of mutagenesis data and understanding structure-activity relationships of allosteric modulator pharmacophores.

Abbreviations: ADX47273, *S*-(4-fluoro-phenyl)-{3-[3-(4-fluoro-phenyl)-[1,2,4]oxadiazol-5-yl]-piperidin-1-yl}-methanone; AMN082, N,N'-Bis(diphenylmethyl)-1,2-ethanediamine; ATCM, allosteric ternary complex model; BINA, Biphenyl-indanone A; Br-5MPEPy, 2-(2-(5-bromopyridin-3-yl)ethynyl)-5-methylpyridine; CaSR, Calcium-sensing receptor; CDPPB, 3-cyano-*N*-(1,3-diphenyl-1*H*-pyrazol-5-yl)benzamide; CFMMC, 3-cyclohexyl-5-fluoro-6-methyl-7-(2-morpholin-4-ylethoxy)-4*H*-chromen-4-one; CPCCOEt, 7-(Hydroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester; CPPHA, *N*-{4-chloro-2-[(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)methyl]phenyl}-2-hydroxybenzamide; DFB, [(3-Fluorophenyl)methylene]hydrazone-3-fluorobenzaldehyde; EM-TBPC, 1-ethyl-2-methyl-6-oxo-4-(1,2,4,5-tetrahydro-benzo[d]azepin-3-yl)-1,6-dihydro-pyrimidine-5-carbonitrile; ERK1/2, extracellular signal-regulated kinases 1 and 2; FMRP, fragile X mental retardation protein; FTIDC, 4-[1-(2-fluoropyridin-3-yl)-5-methyl-1H-1,2,3-triazol-4-yl]-N-isopropyl-N-methyl-3,6-dihydropyridine-1(2*H*)-carboxamide; FXS, Fragile X Syndrome; GABA, γ-aminobutyric acid; GPCR, G protein-coupled receptor; mGlu, metabotropic glutamate receptor; LY404039, (-)-(1R,4S,5S,6S)-4-amino-2-sulfonylbicyclo[3.1.0]hexane-4,6-dicarboxylic acid; LY456066, (2-[4-(indan-2-ylamino)-5,6,7,8-tetrahydro-quinazolin-2-ylsulfanyl]-ethanol hydrochloride); LY487379, 2,2,2-Trifluoro-N-[4-(2-methoxyphenoxy) phenyl]-N-(3-pyridinylmethyl)ethanesulfonamide; M-5MPEP, 2-(2-(3-methoxyphenyl)ethynyl)-5-methylpyridine; M-MPEP, 2-methyl-6-(3-methoxyphenyl)ethynyl-pyridine; MMPIP, 6-(4-Methoxyphenyl)-5-methyl-3-(4-pyridinyl)-isoxazolo [4,5-c] pyridine-4(5*H*)-one hydrochloride; MPEP, 2-Methyl-6-(phenylethynyl)pyridine; MTEP, 3-[(2-Methyl-1,3-thiazol-4-yl)ethynyl] pyridine; NAM, negative allosteric modulator; NMDA, N-methyl-D-aspartate; PAM, positive allosteric modulator; PCP, Phencyclidine; PD, Parkinson's Disease; PET, positron emission tomography; PHCCC, N-Phenyl-7-(hydroxyimino)cyclopropa[b] chromen-1a-carboxamide; R214127, 1-(3,4-dihydro-2H-pyrano[2,3-b]quinolin-7-yl)-2-phenyl-1-ethanone; Ro 67-7476, (S)-2-(4-fluorophenyl)-1-(toluene-4-sulfonyl)pyrrolidine; S-4C3H-PG, (S)-4-carboxy-3-hydroxyphenylglycine; SAR, structure-activity relationship; SIB-1757, 6-Methyl-2-(phenylazo)-3-pyridinol; SIB-1893, 2-Methyl-6-(2-phenylethenyl)pyridine; TM, transmembrane; VFD, Venus-Flytrap domain; VU0155041, cis-2-{[(3,5-Dichlorophenyl)amino]carbonyl} cyclohexanecarboxylic acid; VU29, 4-nitro-N-(1,3-diphenyl-1H-pyrazol-5-yl)benzamide; VU48, 4-nitro-*N*-(1-(2-bromophenyl)-3-phenyl-1*H*-pyrazol-5-yl)benzamide; VU71, 4-nitro-*N*-(1,4-diphenyl-1*H*-pyazol-5-yl)benzamide; YM298198, 6-amino-N-cyclohexyl-N,3-dimethylthiazolo[3,2-a]benzimidazole-2-carboxamide.

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1. Introduction

In addition to eliciting fast excitatory synaptic responses, the neurotransmitter glutamate can modulate neuronal excitability, synaptic transmission, and other cell functions by activation of metabotropic glutamate receptors (mGlus). Due to the ubiquitous distribution of glutamatergic synapses and the broad range of functions of the mGlus, members of this receptor family participate in many different processes in the central nervous system (CNS). As such, mGlus are an attractive target for therapeutic intervention for a range of neurological and psychiatric disorders. mGlus are members of the G protein-coupled receptor (GPCR) superfamily, the largest class of cell-surface receptors. Despite their tractability as drug targets, the majority of GPCR-based drug discovery programs have failed to yield highly selective compounds. The traditional approach to drug discovery has been to target the endogenous ligand (orthosteric)-binding site, to either mimic or block the actions of the endogenous neurotransmitter or hormone in a competitive manner. However, this approach has suffered from a paucity of suitably subtype-selective ligands. This is not surprising given that orthosteric binding sites are often highly conserved between subtypes of a single GPCR subfamily. An alternative approach is to target allosteric sites that are topographically distinct from the orthosteric site, to either enhance or inhibit receptor activation. This approach has been highly successful for ligand-gated ion channels. For example benzodiazepines, positive allosteric modulators (PAMs) of $GABA_A$ receptors, are an effective and safe treatment for anxiety and sleep disorders (Mohler et al., 2002). Discovery and characterization of allosteric modulators of GPCRs has gained significant momentum over the last few years, especially since the clinical validity of GPCR allosteric modulators was demonstrated with two allosteric modulators entering the market. In 2004, cinacalcet (an allosteric enhancer of the Calcium-sensing receptor (CaSR)) was approved for the treatment of hyperparathyroidism, a disease associated with CaSR deficiency (Lindberg et al., 2005). In 2007, maraviroc (an allosteric inhibitor of the chemokine receptor CCR5) was approved for the treatment of HIV infections. This drug stabilizes CCR5 receptor conformations that have a lower affinity for the HIV virus, blocking CCR5-dependent entry of HIV-1 into cells (Dorr et al., 2005). Thus, allosteric modulation represents an exciting novel means of targeting GPCRs particularly for CNS disorders, a therapeutic area with one of the highest rates of attrition in drug discovery (Kola and Landis, 2004).

2. Allosteric modulation of metabotropic glutamate receptors

2.1. Quantifying allosteric interactions

The binding of an allosteric ligand to its site will change the conformation of the receptor, meaning that the "geography" of the orthosteric site and any other potential receptor-ligand/protein interfaces, also have the potential to change. As a consequence, the binding affinity and/or signaling efficacy of the orthosteric ligand are likely to be modulated, either in a positive or negative manner. The simplest allosteric GPCR model assumes that the binding of an allosteric ligand to its site modulates only the affinity of the orthosteric ligand and vice versa; this model is referred to as the allosteric ternary complex model (ATCM; Fig. 1A). Within the framework of an ATCM, the interaction is governed by the concentration of each ligand, the equilibrium dissociation constants of the orthosteric and allosteric ligands (K_A and K_B, respectively), and the "cooperativity factor" α, a measure of the magnitude and direction of the allosteric interaction between the two conformationally linked sites (Stockton et al., 1983; Ehlert, 1988). A value of $\alpha < 1$ (but greater than 0) indicates negative cooperativity, such that the binding of an allosteric ligand inhibits the binding of the orthosteric ligand. Values of $\alpha > 1$ indicate positive cooperativity, such that the allosteric modulator promotes the binding of orthosteric ligand, whereas values of $\alpha = 1$ indicate neutral cooperativity, i.e. no net change in binding affinity at equilibrium. Because the two sites are conformationally linked, the allosteric interaction is reciprocal, i.e., the orthosteric ligand will modulate the binding of the allosteric ligand in the same manner and to the same extent.

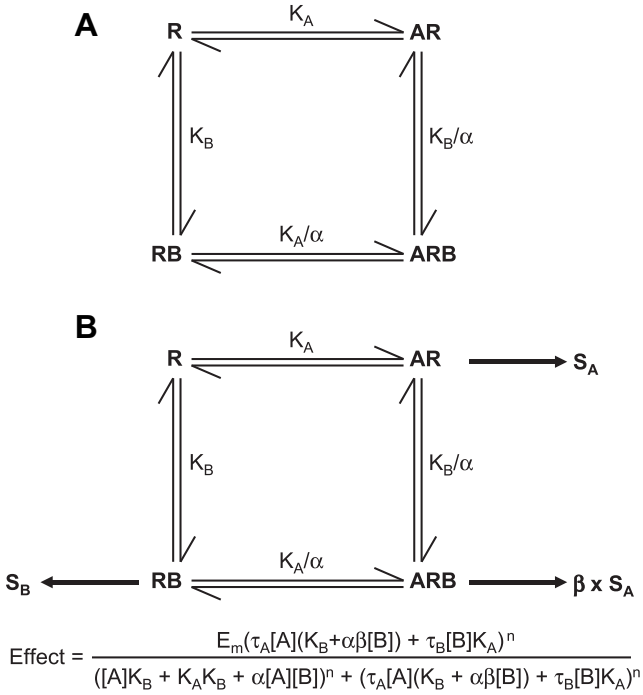

Fig. 1. Models of allosteric interactions. A) Allosteric Ternary Complex Model, B) Operational Model of Allosterism. Refer to text for definitions of parameters.

The simple ATCM describes the effect of the modulator only in terms of changes in orthosteric ligand affinity, and vice versa, thus the stimulus that is generated by the ARB ternary complex (a receptor (R) simultaneously occupied by both agonist (A) and modulator (B)) is assumed to be no different to that imparted by the binary AR complex. In general, many allosteric modulators studied to date, particularly those interacting with class A GPCRs, appear to behave in a manner consistent with this simple ATCM. However, there is no *a priori* reason why the conformational change engendered by an allosteric modulator in the GPCR does not perturb signaling efficacy in addition to, or independently of, any effects on orthosteric ligand binding affinity. Indeed, for mGlus the majority of allosteric modulators influence orthosteric ligand efficacy in the absence of effects on affinity. This is most likely a reflection of the fact that the orthosteric and allosteric binding sites are located in very distinct regions of the receptor i.e. the extracellular N-terminus and the transmembrane-spanning domains respectively (Conn et al., 2009a,b; see later for discussion). It is also important to note that an allosteric modulator can have differential effects on affinity versus efficacy. A striking example of this is the cannabinoid CB_1 receptor allosteric modulator, Org27569, which is an allosteric enhancer of [^{3}H]CP 55940 binding but an allosteric inhibitor of CP 55940 function (Price et al., 2005). This potential for differential effects on efficacy as well as affinity has necessitated the development of alternative models to describe allosteric interactions.

To account for such allosteric effects on efficacy, the ATCM has been extended into an allosteric "two-state" model (ATSM)

(Hall, 2000). An alternate extension of the ATCM has been proposed by Parmentier and colleagues to model the functioning of class C GPCRs (Parmentier et al., 2002). This model accounts for the fact that class C GPCRs have very distinct ligand binding and effector coupling domains and proposes an allosteric interaction between these two domains. While these models provide a framework to describe the vast array of allosteric modulator effects on receptor binding and functional properties, they are not amenable to fitting to experimental biological data due to the large number of model parameters. Recently an "operational model of allosterism" was reported that more simply describes both allosteric modulation of affinity and efficacy and incorporates allosteric agonism (Leach et al., 2007; May et al., 2007b). The operational model of allosterism (Fig. 1B) combines the simple ATCM with an operational model of agonism (Black and Leff, 1983). According to the model, the pharmacological response, initiated by the stimulus (S), can be the result of three different receptor species: the agonist bound (AR), the modulator bound (BR) and the ternary complex (ARB). As for the simple ATCM, allosteric modulation of binding affinity is governed by the cooperativity factor α, whilst allosteric modulation of efficacy is incorporated into the model by the introduction of another parameter, β. The parameters τ_A and τ_B relate to the ability of the orthosteric and allosteric ligands, respectively, to engender receptor activation. Both τ_A and τ_B incorporate the intrinsic efficacy of each ligand, the total density of receptors and the stimulus-response coupling efficiency of the system under investigation. The parameters E_m and n denote the maximal possible system response and the slope factor of the transducer function that links occupancy to response, respectively.

In this operational model of allosterism, allosteric modulation is governed by two cooperativity parameters, α and β, which can vary for each and every set of interacting ligands at a GPCR. However, these should not change for a given set of ligands and GPCR between different assays of GPCR function. An important caveat to this is the potential for pathway specific modulation, in which case the β values will change. It should also be noted that the τ values are determined not only by the individual ligands but also by the biological assay system under investigation, and thus can change between different systems. Analyzing an effector pathway that has low stimulus-response coupling efficiency or alternatively a very low level of receptor expression can yield a low τ value for an agonist and, as such, its efficacy may not be discernible; if the compound is allosteric the interaction will manifest primarily as a change in the potency or maximal response to orthosteric ligand, with no effect on basal signaling. As the τ_B value approaches 0, the species RB becomes equivalent to R and the operational model of allosteric modulation is essentially the ATCM, incorporating efficacy modulation with respect to the species ARB. In the case of receptor over-expression or high stimulus-response coupling efficiency and subsequently high τ_B values, the allosteric ligand efficacy will substantially increase the basal responsiveness of the assay system, and may also shift the orthosteric agonist potency. However, enhancement of the maximal response may not be evident, as a GPCR that has high coupling efficiency may already be approaching the maximal possible response of the entire cellular system (E_m). These are important considerations in terms of designing screening strategies for allosteric ligand-based drug discovery programs, interpreting the pharmacology of putative allosteric modulators and also translating research from recombinant systems to tissues and beyond.

2.2. Structural features of metabotropic glutamate receptors

Arguably, one of the most well studied GPCR families with respect to allosteric modulation are the mGlus. Indeed, the full spectrum of allosteric ligands has been described for these receptors. There are eight mGlu subtypes that are classified into three major groups based on sequence homology, pharmacological properties, and coupling to different second-messenger pathways. Group I includes $mGlu_1$ and $mGlu_5$; group II, $mGlu_2$ and $mGlu_3$; and group III, $mGlu_4$, $mGlu_6$, $mGlu_7$ and $mGlu_8$. mGlus of the same group show ~70% sequence identity whereas between groups this percentage falls to ~45% (Conn and Pin, 1997). Group I mGlus preferentially couple to activation of the $G_{q/11}$ family of G proteins activating phosphoinositide hydrolysis as the major signaling mechanism. In contrast, group II and group III mGlus preferentially couple to $G_{i/o}$ and inhibition of adenylyl cyclases. Members of each group have a unique pharmacological profile and can be selectively activated by specific agonists or allosteric modulators that have no effects on members of the other groups.

GPCRs are predicted to share a common topology consisting of seven transmembrane-spanning α-helical domains, an extracellular N terminus and intracellular C terminus. The mGlus are sub-classified into class C GPCRs along with Calcium-sensing, $GABA_B$, pheromone and taste receptors. Most class C GPCRs are distinguished by their large extracellular N-terminal domain, termed the Venus Flytrap domain (VFD), that contains the endogenous ligand-binding site (Pin et al., 2003). The crystal structures of the N-terminal domains of $mGlu_1$, $mGlu_3$ and $mGlu_7$ suggest that the VFD of the mGlus is made up of two lobes (Kunishima et al., 2000; Tsuchiya et al., 2002; Muto et al., 2007). This forms a clam shell-shaped structure, with the glutamate binding site residing between the two lobes. Evidence suggests that the mGlus dimerize via interactions between their VFDs (Romano et al., 1996). When glutamate binds, the globular domains close into a stable conformation with glutamate inside (Bessis et al., 2000, 2002; Kunishima et al., 2000; Tsuchiya et al., 2002). The conformation changes induced by glutamate binding at the VFD are transmitted via a cysteine-rich domain. The cysteine-rich domain, unique to class C GPCRs (with the exception of the $GABA_B$ receptor which does not have one), links the VFD to the transmembrane-spanning α-helices by a conserved disulfide bridge, subsequently promoting coupling to intracellular G proteins and activation of second messenger pathways (Liu et al., 2004; Rondard et al., 2006; Muto et al., 2007).

While the extracellular N-terminal domain of several mGlus has been crystallized, the structure of the hepta-helical transmembrane domain of the receptor has yet to be determined. In the absence of a crystal structure, homology modeling with class A GPCR templates has been shown to provide substantial insight into the transmembrane region of mGlus. Despite the low sequence identity (less than 20%) between the different classes of GPCRs, confirmation of a common hepta-helical architecture in the mGlu transmembrane region provides support for the use of class A templates as a starting point for homology modeling (Bhave et al., 2003). Differences between GPCR classes prevent homology models from providing structural information at atomic resolution, therefore modeling best occurs synergistically alongside experimental studies of allosteric modulators (Ballesteros and Palczewski, 2001). Early models of $mGlu_1$ and $mGlu_5$ (Ott et al., 2000; Pagano et al., 2000) were based on an alpha-carbon template constructed from a sequence analysis of the transmembrane helices in the rhodopsin family of GPCRs (Baldwin et al., 1997). After the 2.8 angstrom resolution structure of bovine rhodopsin was crystallized (Palczewski et al., 2000), homology models of class C GPCRs revealed possible binding modes of known allosteric modulators within the transmembrane domain of the receptor, which will be discussed in detail below (Ott et al., 2000; Malherbe et al., 2003a,b; Miedlich et al., 2004; Vanejevs et al., 2008). Since the bovine rhodopsin crystal structure, two additional mammalian GPCR crystal structures have become available: the human β_2-adrenergic receptor (Cherezov et al., 2007; Rasmussen et al., 2007; Rosenbaum et al., 2007) and the human A_{2A} adenosine receptor (Jaakola et al., 2008). The growing number

of available templates has sparked development of high-throughput homology modeling of GPCRs (Yarnitzky et al., 2010) and will enrich the understanding of the transmembrane region of these receptors.

2.3. Verifying allosteric modulation through metabotropic glutamate receptor constructs

One way of determining that a putative allosteric modulator is not binding the orthosteric site of a mGlu is through the use of chimeric receptors. Chimeric receptors are often constructed by exchanging the VFD of the receptor for which a modulator is selective with that of a different subtype that the ligand does not affect. If the ligand remains active at the chimeric construct, its activity must then be localized to the transmembrane region or C-terminal region, i.e. an allosteric site. Chimeric receptors were first used to determine agonist selectivity of orthosteric ligands (Takahashi et al., 1993; Tones et al., 1995) and have now become increasingly useful for determining an allosteric mode of action. Interestingly, CPCCOEt was characterized as an allosteric modulator of $mGlu_1$ using a chimeric CaSR and $mGlu_1$ construct (Brauner-Osborne et al., 1999) as well as using chimeras with other mGlus (Litschig et al., 1999; Gasparini et al., 2001). This strategy has proved effective for characterization of numerous negative allosteric modulators (NAMs) and PAMs, with chimeric receptor constructs often used as the first step in the validation of an allosteric mechanism (Pagano et al., 2000; Carroll et al., 2001; Knoflach et al., 2001; Maj et al., 2003; Mitsukawa et al., 2005).

Another method of confirming allosteric binding is to eliminate the extracellular VFD from the receptor altogether through the construction of a 'headless' mGlu. The headless receptor lacks the N-terminal extracellular VFD but retains an intact transmembrane region and a functional C terminus (Goudet et al., 2004). Headless mGlus behave like wild type receptors in terms of G protein coupling and can be positively or negatively regulated by ligands, like any other class A GPCR (Goudet et al., 2004), however, they no longer respond to orthosteric ligands. Allosteric modulators retain activity in cells expressing the headless receptor, PAMs are agonists and NAMs become inverse agonists, as such these constructs are useful tools to identify allosteric ligands (Chen et al., 2007). The headless construct of $mGlu_5$ has been used to localize the binding site of the $mGlu_5$ allosteric modulators MPEP, VU29 and CPPHA to the transmembrane domain (Chen et al., 2007, 2008). FTIDC, an $mGlu_1$ NAM, was also shown to bind to an allosteric site through the use of headless $mGlu_1$ (Suzuki et al., 2007). Chimeric and headless receptors constructs are useful for delineating the location of allosteric binding sites and investigating allosteric interactions, however, they do not provide detailed structural information.

Localization of an allosteric modulator's activity can be narrowed down further to functionally important residues and binding determinants using site-directed mutagenesis (see later for further discussion). Such efforts have been greatly facilitated by the development of radioligands for mGlu allosteric sites. The selective $mGlu_5$ radioligands [^{3}H]-M-MPEP (Gasparini et al., 2002), [^{3}H] methoxy-PEPy (Cosford et al., 2003a,b) and [^{3}H]-methoxymethyl-MTEP (Cosford et al., 2003a,b), provide the opportunity for the characterization of the MPEP binding site on $mGlu_5$. Two radioligands selective for $mGlu_1$ have also been developed, including [^{3}H]R214127 (Lavreysen et al., 2003) and [^{3}H]EM-TBPC (Malherbe et al., 2003a). While second messenger assays are useful for probing the functional effect of a mutation on the interaction between an orthosteric agonist and allosteric modulator, radioligand binding based studies can be used to quantify the influence of a mutation on the affinity of an allosteric modulator. In addition, inhibition binding experiments can be used to determine if a novel allosteric modulator is competitive for known allosteric sites.

2.4. Advantages of allosteric modulation

Allosteric modulators theoretically offer a number of advantages over competitive (orthosteric) agonists and antagonists. Allosteric modulators that have no agonist activity in their own right are quiescent in the absence of the endogenous agonist and will only modulate the receptor once the endogenous agonist is present, thereby retaining spatial and temporal aspects of endogenous receptor signaling. Such 'fine-tuning' of the physiological response is likely to have a better therapeutic outcome than the sustained blockade or activation achieved by orthosteric agonists. A second advantage is the potential for greater subtype selectivity due to either interaction with sites that show greater divergence between subtypes compared to the orthosteric site, or due to selective cooperativity at a particular subtype at the exclusion of others (Lazareno et al., 2004). Another means of generating selectivity is to combine orthosteric and allosteric moieties with the same molecule yielding a bitopic (also referred to as dualsteric) ligand (Valant et al., 2008; Antony et al., 2009). Furthermore, modulators with limited cooperativity, such as the $mGlu_5$ NAMs M-5MPEP and Br-5MPEPy (Rodriguez et al., 2005), will have an in-built "ceiling" level to their effect, suggesting that they may be potentially safer than orthosteric ligands if administered in very large doses. For negative allosteric modulators with limited cooperativity this also introduces the capability to 'dial down' receptor activity, maintaining a residual level of receptor activation, which may in fact be a more desirable therapeutic endpoint than complete blockade. Clearly, allosteric modulators offer a number of advantages over their orthosteric counterparts, although both PAMs and NAMs rely upon the presence of the endogenous ligand. It is also worth noting that drug discovery programs centered on small molecules, be it orthosteric or allosteric, share common problems concerning solubility and formulation, generation of active metabolites, clearance and lack of brain penetrance.

In recent years it has become increasingly evident that the consequences of receptor activation are not limited to G protein activation and subsequent downstream second messengers. Thus, depending upon the measure of receptor activation being employed, ligand pharmacology described can differ. This phenomenon has been given many names including 'stimulus trafficking', 'biased agonism' and 'functional selectivity' (Urban et al., 2007; Kenakin, 2007; Galandrin et al., 2007). Given that allosteric modulators engender unique receptor conformations it is perhaps not surprising that there is the potential for the pharmacology of allosteric modulators to differ dramatically depending upon the assay of receptor activity. For example, MMPIP a negative allosteric modulator of $mGlu_7$, shows differential effects on the receptor activation depending upon the measure of receptor activation and cellular background (Niswender et al., 2010).

It is also becoming increasingly evident that allosteric modulators for a variety of GPCRs can not only modulate orthosteric ligand signaling, but also act as agonists in their own right (Spalding et al., 2002, 2006; Sachpatzidis et al., 2003; Mitsukawa et al., 2005; Zhang et al., 2005; Langmead et al., 2006; Nawaratne et al., 2008; Tu et al., 2007; Pelkey et al., 2007; Lee et al., 2007; May et al., 2007a; Jones et al., 2008; Niswender et al., 2008; Holst et al., 2009; Lebois et al., 2010). These so-called allosteric agonists add an additional layer of complexity and even more scope for treatment options. It should also be noted that there is no reason why a modulator could not express more than one of these properties concomitantly, e.g., agonism (positive or inverse) together with enhancement or inhibition of orthosteric ligand binding/function (Schwartz and Holst, 2007; May et al., 2007b). If a particular pathway can be associated with the pathophysiology of a disease or therapeutic improvement, then selective activation or inhibition of particular pathways by allosteric

ligands may also represent a novel means of altering receptor activation. Currently, it remains to be determined whether a single phenotype (modulator only) or a combination of both modulator and agonist properties is the optimal approach to treating GPCR-based diseases with allosteric ligands. Most likely, different therapies will benefit differently from one phenotype relative to another.

3. Physiological roles of metabotropic glutamate receptors

With the exception of $mGlu_6$ which is localized to the retina, mGlus are ubiquitously expressed throughout the CNS in both neurons and glia, although each subtype is differentially localized in different brain regions (specific brain localizations for each of the mGlu subtypes is reviewed in detail in Ferraguti and Shigemoto, 2006). In recent years, all mGlu subtypes have been genetically deleted in mice; studies using these animals have yielded further insights into the biological functions of mGlu as well as potential diseases where mGlus may be a viable target for therapeutic intervention (see Niswender and Conn, 2010 for review). Detailed discussion of the physiological roles of mGlus has been presented in multiple reviews (Coutinho and Knopfel, 2002; Conn and Pin, 1997; Anwyl, 1999; Valenti et al., 2002; Bellone et al., 2008; Pinheiro and Mulle, 2008). Elucidation of these roles for mGlus suggests that selective activators and inhibitors of specific mGlu subtypes could subtly alter transmission in glutamatergic circuits in a therapeutically beneficial manner without eliciting the side effects commonly associated with drugs that interact with members of the ionotropic glutamate receptor family.

While it is clear that the specific mGlu subtype involved in mediating a given effect varies in different brain regions, some generalizations can be made regarding the common functions of different groups of mGlus. Group I mGlus are generally found postsynaptically, whilst group II and III mGlus are often localized on presynaptic terminals or preterminal axons. Activation of postsynaptic group I mGlus often leads to cell depolarization and increases in neuronal excitability via modulation of a variety of ion channels. This modulation can range from robust excitation to more subtle changes in the pattern and frequency of cell firing and responses to excitatory inputs (Coutinho and Knopfel, 2002; Anwyl, 1999; Valenti et al., 2002). Presynaptic group II and III mGlus inhibit neurotransmitter release on a variety of excitatory (glutamatergic), inhibitory (GABAergic) and neuromodulatory (monoamines, ACh, peptides) synapses.

4. Therapeutic indications for allosteric modulators of metabotropic glutamate receptors

The first allosteric modulator of an mGlu identified was CPCCOEt, a NAM of $mGlu_1$ (Annoura et al., 1996). Selective promising allosteric modulators have been identified for many mGlu subtypes (see Fig. 2 and Table 1) and are exciting potential therapeutics for a variety of CNS-related disorders including Alzheimer's disease (Lee et al., 2004), anxiety disorders (Spooren and Gasparini, 2004; Swanson et al., 2005), depression (Palucha and Pilc, 2007), epilepsy (Alexander and Godwin, 2006; Ure et al., 2006), and Parkinson's disease (Conn et al., 2005; Johnson et al., 2009) among others. Comprehensive reviews encompassing each of these therapeutic areas are available, however, several areas warrant mentioning as they signify important advances in the field.

4.1. Negative allosteric modulation of metabotropic glutamate receptor 5 for anxiety and Fragile X Syndrome

In brain regions implicated in the pathology of anxiety disorders, such as the amygdala, $mGlu_5$ is localized at postsynaptic sites where it increases the excitability of the *N*-methyl-D-aspartate (NMDA) subtype of ionotropic glutamate receptors (Romano et al., 1995; Valenti et al., 2002). Based on this physiology, it has been hypothesized that antagonists of $mGlu_5$ might reduce the activity of glutamatergic synapses that are thought to contribute to the underlying mechanisms of anxiety disorders (Marino and Conn, 2006). Indeed, a role for group I mGlus in anxiety was indicated by the finding that intrahippocampal administration of the group I antagonist (S)-4-carboxy-3-hydroxyphenylglycine (S-4C3H-PG) had anxiolytic properties using the conflict drinking Vogel test in rats (Chojnacka-Wójcik et al., 1997). However, S-4C3H-PG also possesses partial agonism at group II mGlus (Hayashi et al., 1994). $mGlu_1$ selective antagonists, such as JNJ16259685, also showed efficacy in rodent models of anxiety, however, these compounds were associated with memory impairments that prohibited further development (Steckler et al., 2005a,b; Gravius et al., 2005; Pietraszek et al., 2005). Fortunately, $mGlu_5$ selective antagonists whilst being anxiolytic, did not cause the same degree of impairments in memory (Steckler et al., 2005a; Gravius et al., 2005; Pietraszek et al., 2005). The first identified selective NAMs of $mGlu_5$ were SIB-1757 and SIB-1893 and subsequent structural analogues MPEP and MTEP have been developed that possess improved potency, selectivity and brain penetrance (Varney et al., 1999; Gasparini et al., 1999; Cosford et al., 2003a). There are now a large number of highly selective $mGlu_5$ NAMs, including radioligands and positron emission tomography (PET) ligands (Gasparini et al., 2002; Anderson et al., 2002, 2003; Cosford et al., 2003b; Ametamey et al., 2007; Treyer et al., 2008; Yu, 2007; Baumann et al., 2010). The availability of radioligands and PET ligands may prove useful for dose-finding studies for NAMs in clinical development as they provide the necessary tools to assess receptor occupancy.

The availability of selective and systemically active $mGlu_5$ NAMs has allowed for the validation of $mGlu_5$ inhibition as a viable therapeutic strategy for anxiety disorders. In multiple rodent models of anxiety, MPEP and related compounds have been shown to be anxiolytic (Spooren et al., 2000, 2002; Schulz et al., 2001; Tatarczynska et al., 2001; Rodrigues et al., 2002). Recently, fenobam, a non-benzodiazepine anxiolytic, was found to be a selective $mGlu_5$ NAM (Porter et al., 2005). Fenobam is efficacious in preclinical rodent models of anxiety (Patel et al., 1982; Goldberg et al., 1983; Porter et al., 2005) and in clinical trials (Pecknold et al., 1982). Collectively, these data support the hypothesis that negative allosteric modulation of $mGlu_5$ is an attractive avenue for the development of novel anxiolytics.

Fragile X Syndrome (FXS) is the leading cause of autism and the most common form of inherited mental retardation (Crawford et al., 2001; Garber et al., 2008). FXS is caused by a mutation in the gene encoding fragile X mental retardation protein (FMRP), which represses the translation of specific mRNAs regulating protein translation in neuronal dendrites. In mice, a loss of FMRP results in increased group I mGlu-dependent long-term depression in the hippocampus (Huber et al., 2002). Administration of MPEP to FXS mice reduces anxiety and seizures (Yan et al., 2005), whilst crossbreeding of FXS mice with $mGlu_5$ knock-out mice suggest that a number of fragile X phenotypes can be corrected by reducing levels of $mGlu_5$ (Dolen et al., 2007). Indeed, fenobam treatment improved clinical behaviors and prepulse inhibition with no adverse effects in adults with FXS (Berry-Kravis et al., 2009). Furthermore, AFQ056, another $mGlu_5$ NAM is in phase 2 clinical trials for adults with FXS (Novartis, 2010a). Therefore, antagonists of $mGlu_5$ represent promising novel treatment strategies for FXS (Bear et al., 2008).

In addition to anxiety and FXS, $mGlu_5$ NAMs have also been suggested as treatment options for iatrogenic dystonias, substance

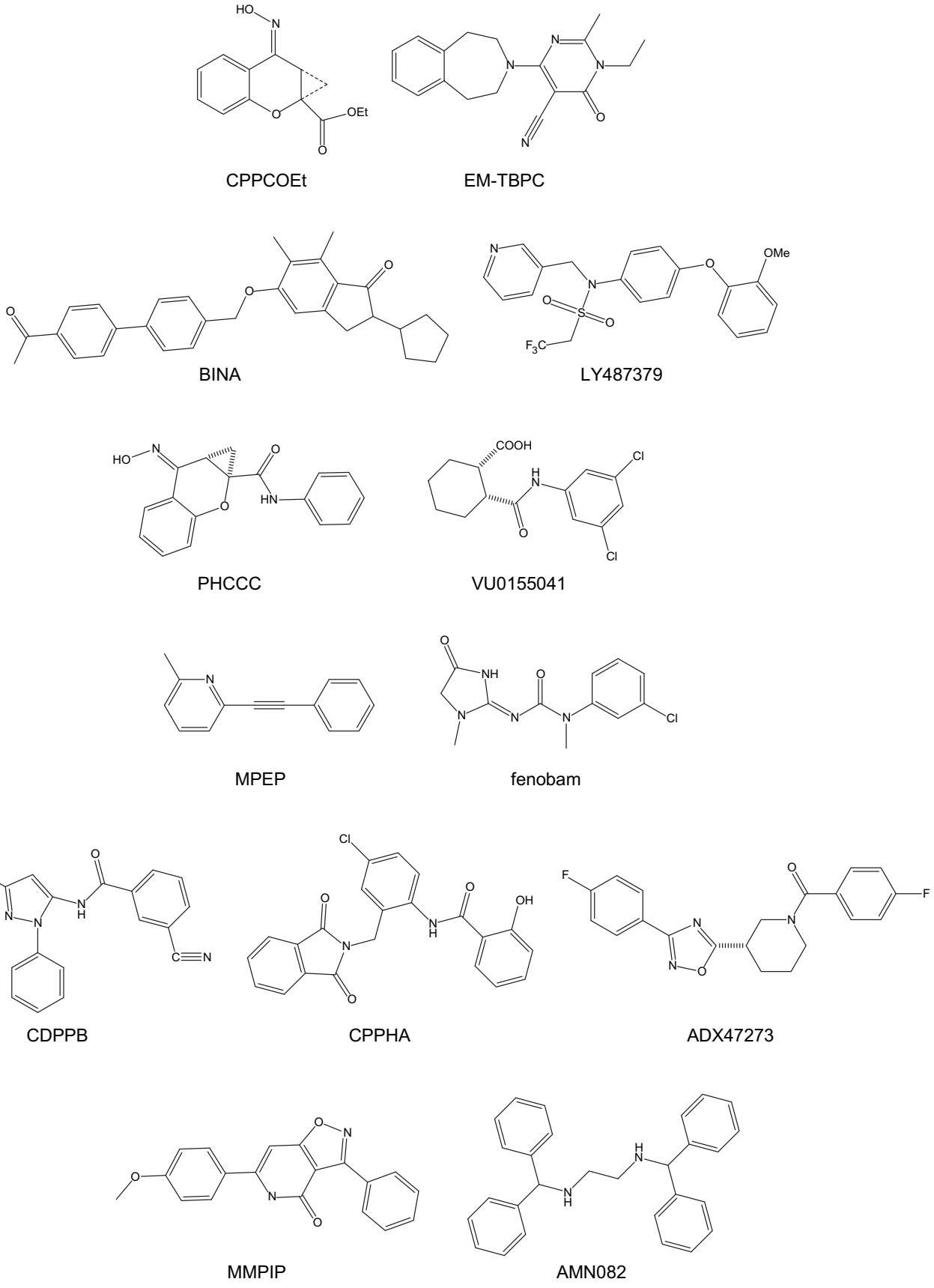

Fig. 2. Prototypical allosteric modulators of mGlus. Pictured are representative allosteric modulators for $mGlu_1$, $mGlu_2$, $mGlu_4$, $mGlu_5$ and $mGlu_7$. The full chemical names and subtype selectivity of these compounds are provided in Table 1.

Table 1
Therapeutic indications for metabotropic glutamate receptors in CNS disorders and examples of allosteric modulators.

mGlu subtype	Therapeutic indication	Intervention	Representative allosteric modulators	
			Compound	Chemical name
1	Pain	NAM	CPCCOEt	7-(Hydroxyimino)cyclopropa[*b*]chromen-1a-carboxylate ethyl ester
			EM-TBPC	1-ethyl-2-methyl-6-oxo-4-(1,2,4,5-tetrahydro-benzo[d]azepin-3-yl)-1,6-dihydro-pyrimidine-5-carbonitrile
2	Anxiety, schizophrenia	Agonist/PAM	BINA	Biphenyl-indanone A
			LY487379	2,2,2-Trifluoro-N-[4-(2-methoxyphenoxy)phenyl]-N-(3-pyridinylmethyl)ethanesulfonamide
4	Parkinson's disease, movement disorders	Agonist/PAM	PHCCC	N-Phenyl-7-(hydroxyimino)cyclopropa[*b*]chromen-1a-carboxamide
			VU0155041	cis-2-{[(3,5-Dichlorophenyl)amino]carbonyl} cyclohexanecarboxylic acid
5	Anxiety, Fragile X Syndrome, chronic pain, depression, migraine, Parkinson's disease levodopa-induced dyskinesia	NAM	MPEP	2-Methyl-6-(phenylethynyl)pyridine
			Fenobam	*N*-(3-chlorophenyl)-*N*′-(4,5-dihydro-1-methyl-4-oxo-1H-imidazole-2-yl)urea
5	Schizophrenia, cognition disorders	PAM	ADX47273	*S*-(4-fluoro-phenyl)-{3-[3-(4-fluoro-phenyl)-[1,2,4]oxadiazol-5-yl]-piperidin-1-yl}-methanone
			CDPPB	3-cyano-*N*-(1,3-diphenyl-1*H*-pyrazol-5-yl)benzamide
			CPPHA	*N*-{4-chloro-2-[(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)methyl]phenyl}-2-hydroxybenzamide
7	Depression, anxiety	NAM	MMPIP	6-(4-Methoxyphenyl)-5-methyl-3-(4-pyridinyl)-isoxazolo [4,5-c]pyridine-4(5*H*)-one hydrochloride
7	Epilepsy, depression, anxiety	Agonist/PAM	AMN082	*N*,*N*′-Bis(diphenylmethyl)-1,2-ethanediamine
8	Epilepsy, anxiety, drug abuse, pain	Agonist/PAM	No selective allosteric ligands reported	

abuse and withdrawal, chronic pain, depression, gastroesophogeal reflux disorder and migraine (Slassi et al., 2005). AFQ056 is also in phase II clinical trials for L-DOPA-induced dyskinesia (LID) associated with the treatment of Parkinson's disease (PD) (Novartis, 2010b–d; Rylander et al., 2010). Furthermore, the $mGlu_5$ NAM, ADX10059, showed efficacy for the treatment of gastroesophogeal reflux disorder (Keywood et al., 2009; Zerbib et al., 2010), however, a separate trial investigating efficacy in migraine was terminated due to liver toxicity (Addex, 2010).

An important consideration in the clinical development of $mGlu_5$ NAMs is the potential for target-related adverse effects. $mGlu_5$ plays an important role in cognitive processing and administration of MPEP has been shown to exacerbate psychosis induced by PCP (Campbell et al., 2004), therefore $mGlu_5$ NAMs may cause cognitive impairments and psychotomimetic effects. Indeed, psychostimulant adverse effects were reported in early fenobam clinical trials (Itil et al., 1978; Friedmann et al., 1980; Pecknold et al., 1982), although whether or not this was due to interactions with $mGlu_5$, an unknown interaction or through the formation of active metabolites remains to be seen. However, no psychostimulant effects or cognitive impairments were reported for fenobam in a recent trial in FXS patients, nor for another $mGlu_5$ NAM, ADX10059 (Berry-Kravis et al., 2009; Keywood et al., 2009; Zerbib et al., 2010). In a recent study, Rodriguez et al. (2005) reported the discovery of $mGlu_5$ NAMs with limited negative cooperativity. These so-called partial antagonists do not completely block the activity of glutamate, despite achieving 100% receptor occupancy. NAMs with limited cooperativity could have improved clinical success, as they would retain some level of activity at the receptor, which may result in an improved adverse-effect profile.

4.2. Positive allosteric modulation of metabotropic glutamate receptor 5 for schizophrenia and cognitive disorders

Whilst $mGlu_5$ antagonists are an attractive strategy for a number of disorders, positive allosteric modulation of $mGlu_5$ has arisen as exciting new approach for the treatment of schizophrenia and cognitive disorders. Until recently, the neurochemical mechanism underlying schizophrenia has been attributed to hyperactivity of the dopaminergic system. However, evidence has accumulated to suggest that hypofunction of glutamatergic receptors, particularly NMDA receptors, could also contribute to the underlying neurochemical cause of schizophrenia (Conn et al., 2009a). Clinical studies have shown that antagonists of the NMDA receptor, such as PCP and ketamine, induce symptoms in rats and humans that mirror the positive, negative and cognitive symptoms of schizophrenic patients (Gaspar et al., 2009). Conversely, ligands that enhance NMDA receptor function have proven to be efficacious in the treatment of schizophrenia (Lindsley et al., 2006). Interestingly, $mGlu_5$ has been identified as a closely associated signaling partner with NMDA receptors and may play an integral role in regulating NMDA receptor function in brain regions involved in cognitive function and implicated in the pathology of schizophrenia such as the hippocampus, striatum and prefrontal cortex (Alagarsamy et al., 1999, 2005; Ugolini et al., 1999; Ehlers, 1999; Awad et al., 2000; Doherty et al., 2000; Attucci et al., 2001; Mannaioni et al., 2001; Pisani et al., 2001; Marino & Conn, 2002). Therefore, selective activation of $mGlu_5$ and subsequent enhancement of NMDA receptor activity may provide a novel means of improving not only the positive symptoms but also the cognitive impairments associated with schizophrenia. The first selective $mGlu_5$ PAMs include three different chemical series: DFB, CPPHA and CDPPB (O'Brien et al., 2003, 2004; Lindsley et al., 2004; Kinney et al., 2005). All three of these compounds cause a leftward shift in the glutamate concentration response curve of approximately 10 fold for CPPHA and CDPPB and 2–4 fold for DFB, yet have no impact on the affinity of glutamate. It is also worth noting that while DFB and CDPPB and derivatives thereof are thought to interact competitively with MPEP, CPPHA acts at a second distinct allosteric site within the TM domains (see later for details).

The discovery, development and optimization of these PAMs, in particular DFB and CPPHA, highlight some of the challenges faced by medicinal chemists when pursuing allosteric modulators. The structure-activity relationship (SAR) of CPPHA was 'flat', as evidenced

by the fact that out of 995 analogues only 45 were active (Zhao et al., 2007). For DFB the SAR was even more interesting, with slight modifications to the scaffold rendering not only inactive compounds but also causing significant changes in the pharmacology, switching the compounds from PAMs to NAMs or resulting in neutral cooperativity (O'Brien et al., 2003). Whilst the medicinal chemistry efforts surrounding the CDPPB scaffold were more successful, they did not yield any significant improvements overall (de Paulis et al., 2006). Despite these challenges, these initial $mGlu_5$ PAMs provided much needed tools study potentiation of $mGlu_5$ activity in electrophysiological preparations (O'Brien et al., 2003, 2004; Chen et al., 2007; Ayala et al., 2009) as well as in rodent behavioral models (Kinney et al., 2005; Balschun et al., 2006; Lecourtier et al., 2007; Gass and Olive, 2009; Uslaner et al., 2009; Stefani and Moghaddam, 2010). Recently, a fourth PAM chemotype represented by ADX47273 was reported, which also displays efficacy in rodent behavioral models similar to proven antipsychotics and importantly, improved cognitive functioning in impaired animals (Liu et al., 2008; Schlumberger et al., 2009, 2010). Thus, $mGlu_5$ PAMs show much promise as potential therapeutics for the treatment of schizophrenia and also as cognition enhancers.

4.3. Positive allosteric modulation of metabotropic glutamate receptor 2 for schizophrenia and anxiety disorders

There is now a large body of evidence that activation of group II mGlus may present a novel means of treating schizophrenia and anxiety disorders. In brain regions implicated in the pathology of schizophrenia it is hypothesized that hypofunction of NMDA receptors leads to decreased downstream activation of GABAergic neurons resulting in disinhibition and an overall increase in excitation (Swanson et al., 2005; Conn et al., 2009a). Activation of $mGlu_2$ has been shown to decrease excitatory amino acid transmission at a number of synapses (Macek et al., 1996; Doherty et al., 2004; Nicholls et al., 2006). Orthosteric group II mGlu agonists have efficacy in preclinical models of psychosis and anxiety (Moghaddam and Adams, 1998; Cartmell et al., 1999; Lorrain et al., 2003; Swanson et al., 2005; Conn et al., 2008). In addition, there is evidence for improvement in cognitive deficits in both humans (induced by ketamine) and rats (induced by PCP) (Moghaddam and Adams, 1998; Cartmell et al., 1999; Krystal et al., 2005). In a recent clinical trial, LY2140023, an oral prodrug of the orthosteric agonist LY404039, improved the positive and negative symptoms of schizophrenic patients with a similar efficacy to olanzapine, without the side effects associated with typical and atypical antipsychotics (Patil et al., 2007). Currently, LY2140023 is entering phase II/III trials for schizophrenia (Eli Lilly, 2010). Similarly, LY354740 has clinical efficacy in treating panic attacks and generalized anxiety disorder (Schoepp et al., 2003; Schoepp, 2004). Whilst these orthosteric agonists have much promise as therapeutic options, there are a number of disadvantages. These orthosteric agonists activate both $mGlu_2$ and $mGlu_3$, they are all based on a similar chemical scaffold, and tolerance was induced in one rodent model used to assess anti-psychotic efficacy (Galici et al., 2005). Development of tolerance is a key concern for CNS disorders that require long-term treatment as it can result in both a loss of efficacy over time and changes in plasticity leading to adverse effects, as exemplified by the clinically efficacious orthosteric D2 dopamine receptor antagonists (Wadenberg et al., 2001; Natesan et al., 2005; Ginovart et al., 2009). Thus, $mGlu_2$ PAMs are an attractive alternative as they may have the capacity to overcome some of these potential shortcomings.

Numerous $mGlu_2$ selective PAMs have now been reported, the majority of which are related to either BINA or LY487379 (Johnson et al., 2003; Lorrain et al., 2003; Schaffhauser et al., 2003; Cube et al., 2005; Galici et al., 2005, 2006; Pinkerton et al., 2005). Both BINA and LY487379 induce leftward shifts of the glutamate concentration response curve, and potentiate the ability of group II selective agonists to reduce transmission at a number of glutamatergic synapses (Johnson et al., 2003; Schaffhauser et al., 2003; Poisik et al., 2005; Galici et al., 2006; Benneyworth et al., 2007). In rodent behavioral models predictive of anti-psychotic and anxiolytic efficacy, these $mGlu_2$ PAMs have similar efficacy to group II orthosteric agonists (Galici et al., 2005; Govek et al., 2005; Johnson et al., 2005; Pinkerton et al., 2005; Benneyworth et al., 2007). Therefore there is much excitement that selective $mGlu_2$ PAMs represent a novel treatment strategy for schizophrenia and anxiety that will have improved outcomes and side effect profiles over currently used anti-psychotics.

4.4. Positive allosteric modulation of metabotropic glutamate receptor 4 for Parkinson's disease

Parkinson's disease (PD) is a common neurodegenerative disorder characterized by severe motor impairments such as bradykinesia, tremor and rigidity. These motor symptoms arise as a consequence of a progressive loss of dopaminergic neurons in the substantia nigra, which project into the striatum regulating activity through the basal ganglia. Dopamine reduces the activity of striatal neurons projecting into the globus pallidus, in PD patients the loss of dopaminergic neurons results in overactivity of these synapses (Wichmann and DeLong, 2003). Current therapeutics aim to restore dopaminergic modulation, such as administration of L-DOPA, the precursor of dopamine. However, dopamine replacement therapy is associated with increased adverse effects as the disease progresses and a loss of efficacy (Poewe et al., 1986a,b). Therefore alternative therapeutic options are much in demand. At the striato-pallidal synapse, activation of $mGlu_4$ reduces neurotransmission (Matsui and Kita, 2003; Valenti et al., 2003; Conn et al., 2005; MacInnes and Duty, 2008). In addition, the $mGlu_4$ preferring orthosteric agonist LSP1-2111 has demonstrated efficacy in rodent models of PD, specifically reversal of haloperidol-induced catalepsy and 6-hydroxydopamine-induced motor deficits (Beurrier et al., 2009). This has been linked to the inhibition of striato-pallidal GABAergic transmission through mGlu4 activation (Cuomo et al., 2009). These studies lend support to the hypothesis that $mGlu_4$ PAMs or allosteric agonists are a viable approach to the treatment of the symptoms of PD.

The $mGlu_5$ NAMs SIB-1893 and MPEP are also positive allosteric modulators of $mGlu_4$, potentiating the response to L-2-amino-4-phosphonobutyrate, an orthosteric agonist (Mathiesen et al., 2003). The first selective $mGlu_4$ PAM, PHCCC, was recently identified (Flor et al., 2002; Marino et al., 2003; Maj et al., 2003). PHCCC has no agonist activity but increases the potency of glutamate in recombinant systems as well as at several synapses including the striato-pallidal synapse (Marino et al., 2003; Valenti et al., 2005). Moreover, PHCCC decreases reserpine-induced akinesia (Marino et al., 2003), a rodent model of PD, and is neuroprotective in a model of dopaminergic cell death (Battaglia et al., 2006). A novel $mGlu_4$ PAM chemotype, exemplified by VU0155041, was recently identified in a high-throughput screen by Niswender and colleagues (2008). Interestingly, VU0155041 was found to be an allosteric agonist in addition to its PAM properties, with significant advantages over PHCCC, namely improved aqueous solubility, potency and selectivity. VU0155041 was also efficacious in reversing haloperidol-induced catalepsy and reserpine-induced akinesia (Niswender et al., 2008). Clearly, there is much promise for the development of $mGlu_4$ PAMs and allosteric agonists as novel therapeutics for PD.

5. Localization of allosteric sites on metabotropic glutamate receptors

5.1. Common allosteric sites within and between metabotropic glutamate receptors

One of the continuing challenges faced in drug discovery is establishing suitably subtype selective ligands. Although allosteric ligands have better specificity than orthosteric ligands, there are numerous examples of allosteric modulators that interact with multiple receptor subtypes. As mentioned above, MPEP, an $mGlu_5$ NAM, is an $mGlu_4$ PAM (Mathiesen et al., 2003), while DFB and CPPHA ($mGlu_5$ PAMs) are also weak $mGlu_4$ NAMs (O'Brien et al., 2003, 2004). Similarly, PHCCC, an $mGlu_4$ PAM, is also an $mGlu_1$ NAM (Annoura et al., 1996). Although problematic in terms of identifying new chemotypes, this lack of selectivity for some compounds across different mGlu groups suggests similarities in allosteric binding pockets on these receptors.

To identify the sites at which allosteric modulators bind mGlus, initial mutational sites were chosen based on models predicting a pocket that may resemble the site where 11-*cis*-retinal, an inverse agonist, binds in bovine rhodopsin (Palczewski et al., 2000; Teller et al., 2001). Indeed, several critical residues for *cis*-rhodopsin binding accurately predicted residues that are important for binding of mGlu allosteric ligands, validating the use of rhodopsin as a template for the transmembrane region of class C GPCRs (Malherbe et al., 2003a,b). Critical residues for allosteric modulator binding and cooperativity determinants have been characterized for $mGlu_1$, $mGlu_2$ and $mGlu_5$ (Table 2). A complete table of mutational studies on allosteric modulators of mGlus can be found in the supplementary material.

In the characterization of potential binding pockets for mGlu allosteric modulators, within a particular receptor subtype the majority of structurally unrelated PAMs and NAMs tend to cluster in overlapping binding sites. As can be seen from Table 2 and the sequence alignment in Fig. 3, the binding site for [^{3}H]EM-TBPC in $mGlu_1$ is primarily located on TM 6 and 7 (Malherbe et al., 2003a), which is also where functionally important residues are located for other $mGlu_1$ NAMs: CFMMC, LY456066, YM298198, FTICD and CPCCOEt (Litschig et al., 1999; Surin et al., 2007; Suzuki et al., 2007; Fukuda et al., 2009). When mapped onto a 3D model of the heptahelical transmembrane domain (using the backbone coordinates from the β_2-adrenergic receptor X-ray crystal structure) these residues are found on the top half of TMs 3, 5, 6 and 7 on the inside faces of these helices (Fig. 4A).

Key binding determinants for allosteric modulation by MPEP and fenobam at $mGlu_5$ are found on TMs 3, 6 and 7, as seen in Table 2 and Fig. 3 (Pagano et al., 2000; Malherbe et al., 2003b; 2006). Similarly, functionally important residues for positive allosteric modulation by DFB and VU29 show a similar distribution (Muhlemann et al., 2006; Chen et al., 2008). Fig. 4C demonstrates the clustering of these residues on the top half of the TMs, again located on the inside face of the helices. Competition binding assays with radioligands provide additional support for a common binding

Table 2
Functionally critical residues and binding determinants of positive and negative allosteric modulation of mGluR receptors.

	Positive Allosteric Modulators (PAMs)						Negative Allosteric Modulators (NAMs)					
	mGluR1		mGluR2	mGluR5			mGluR1				mGluR5	
7TM position[b]	CPPHA	Ro 67-7476	LY487379, MRLSD-650, BINA	CPPHA	VU-29	DFB	EM-TBPC	CFMMC	LY45606, YM298198 FTICD	CPCCOEt	MPEP	fenobam
TM1												
1.42	F599			F585								
TM3												
3.29											R648	R648
3.36		S668				P655					P655[a]	P655[a]
3.39		C671				S658					S658[a]	S658[a]
3.40							Y672				Y659[a]	Y659[a]
TM4												
4.45			S688									
4.46			G689									
4.55								I725				
EC2												
45.51							N747					
45.54						N734	N750					
TM5												
5.47		L757				L744	L757					L744[a]
5.48			A733[d]									
5.50			N735					N760	N760[c]			
TM6												
6.43						T781					T781	T781[a]
6.47						W785	W798	W798	W798[c]		W785[a]	W785[a]
6.51						F788	F801[a]	F801	F801		F788[a]	F788[a]
6.55						Y792	Y805[a]	Y805	Y805		Y792	Y792[a]
TM7												
7.32						M802	T815[a]	T815	T815	T815	M802[a]	
7.35										A818	S805[a]	
7.40					A810	A810					A810[a]	A810[a]

[a] Residues implicated in binding have been determined using selective allosteric radioligands.
[b] The position of each residue in the mGluR 7TMD is given by the numbering system proposed by Ballesteros and Weinstein (1995), which allows for the comparison of equivalent positions within GPCRs. The first number represents the TM helix and the second number is its position relative to a highly conserved residue in the group A GPCRs from that TM, assigned the number 50. Residues in the extracellular loop E2 are labeled '45' to indicate their location between helix 4 and 5. Highly conserved residues (assigned to position 50) are from the bovine rhodopsin sequence: N55$^{1.50}$, D83$^{2.50}$, R135$^{3.50}$, W161$^{4.50}$, C187$^{45.50}$, P215$^{5.50}$, P267$^{6.50}$, P303$^{7.50}$.
[c] Effects LY456066 only.
[d] BINA not tested at mutations of this residue.

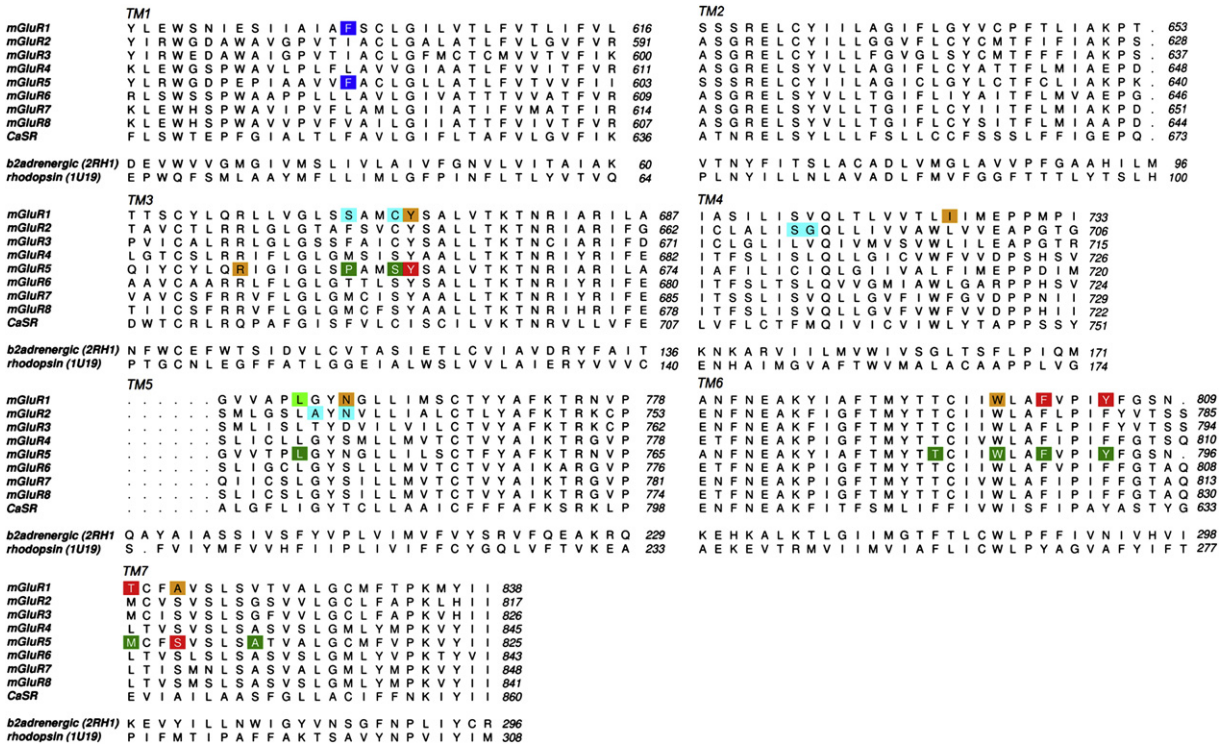

Fig. 3. Alignment of the 7TMD in the human mGlus and Calcium-sensing receptor (CaSR) sequences (aligned with CLUSTALW) relative to β_2-adrenergic receptor (2RH1) and bovine rhodopsin (1U19) sequences (aligned with MUSTANG). Alignment of TM regions between class C GPCRs and bovine rhodopsin were directly adopted from Malherbe et al. (2006), except TM2, 4 and 7, which were based on the alignment of CaSR with bovine rhodopsin from Miedlich et al. (2004). Highlighted are: residues functionally important for CPPHA (blue) or other PAMs (cyan), residues important for NAMs functionally (orange) and through binding (red), and residues important for both PAMs and NAMs functionally (light green) or both PAM function and NAM binding (dark green). The full alignment is provided in the supplementary material (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

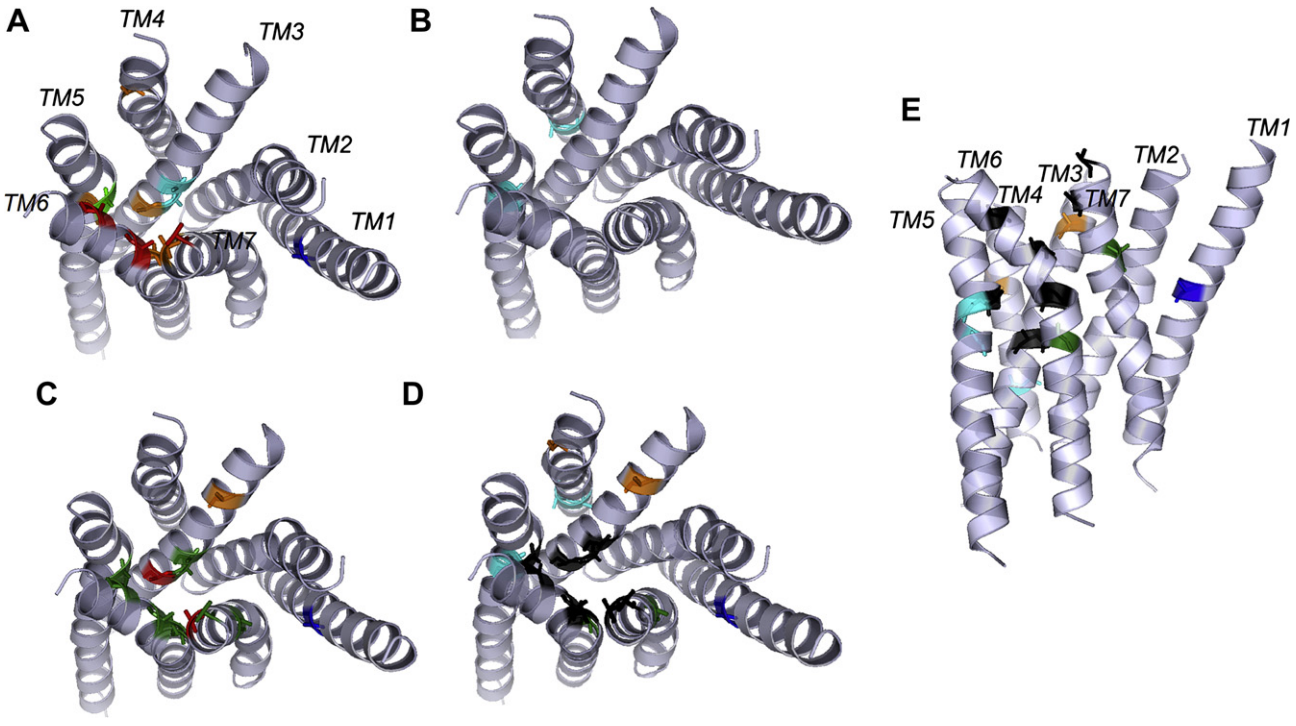

Fig. 4. Functionally critical residues and binding determinants for mGlu allosteric modulators mapped onto the hepta-helical transmembrane domain of β_2-adrenergic receptor X-ray crystal structure (2RH1) backbone. Shown in sticks is the backbone beta-carbon of functionally important residues and binding determinants. A) $mGlu_1$, B) $mGlu_2$, C) $mGlu_5$, D) residues from $mGlu_1$, $mGlu_2$ and $mGlu_5$ and E) side view of residues from $mGlu_1$, $mGlu_2$ and $mGlu_5$. Highlighted are: residues functionally important for CPPHA (blue) or other PAMs (cyan), residues important for NAMs functionally (orange) and through binding (red), and residues important for both PAMs and NAMs functionally (light green) or both PAM function and NAM binding (dark green). In D and E, residues important for both $mGlu_1$ and $mGlu_5$ allosteric modulation are in black (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

site for allosteric modulators. In $mGlu_5$ studies, CDPPB inhibits allosteric binding of the MPEP analog [^{3}H]methoxyPEPy in a competitive manner (Chen et al., 2007). In addition, point mutations reducing the binding of MPEP also decrease the ability of CDPPB to potentiate $mGlu_5$ response to glutamate (Chen et al., 2007). These results suggests that CDPPB, along with its derivative VU29, share a common or overlapping binding site with MPEP (Kinney et al., 2005; Chen et al., 2007).

The allosteric binding sites for the majority of allosteric modulators of group I mGlus are located in similar regions. As seen in Fig. 4D, overlaying the key residues for allosteric modulation from both receptors clearly demonstrates the similarity between the two receptor subtypes. Experiments performing residue swaps provide further evidence for similar binding sites between $mGlu_1$ and $mGlu_5$. Substituting non-conserved residues of $mGlu_1$, known to be important for CPCCOEt and Ro 67-7476 allosteric modulation, onto $mGlu_5$ results in a gain of function for these $mGlu_1$ selective modulators at $mGlu_5$ (Litschig et al., 1999; Knoflach et al., 2001). Similarly, substitution of residues important for MPEP binding onto $mGlu_1$ results in the gain of [^{3}H]MPEP binding (Pagano et al., 2000). Clearly, there is evidence that the location of at least one allosteric site is very similar for the group I mGlus, despite the availability of selective allosteric modulators for these sites. In contrast, the residues identified that perturb modulation by group II selective PAMs are found in TM 4 and 5 (Figs. 3 and 4B) (Schaffhauser et al., 2003; Hemstapat et al., 2007; Rowe et al., 2008). The two residues identified in TM5 cluster near the common allosteric site of group I mGlus, whilst those in TM4 do not. Unlike the mutations in group I mGlus, the existing $mGlu_2$ data relies entirely upon functional assays, thus, it remains to be seen whether these residues are required for modulator binding or the transmission of cooperativity. More studies are required of $mGlu_2$ selective modulators to determine whether or not the binding pocket utilized is similar to that shared by the majority of group I mGlu allosteric modulators.

5.2. Multiple allosteric sites within a single metabotropic glutamate receptor type

While commonalities between allosteric binding sites within subtypes of mGlus have been the prevailing trend, there is evidence for multiple allosteric sites on the same receptor. Inhibition binding assays with radioligands and Schild-like analysis with neutral allosteric ligands has enabled the detection of mGlu allosteric modulators that do not interact with the common binding sites. This has been most definitively shown for CPPHA, a group I PAM. CPPHA does not displace the binding of [^{3}H]methoxyPEPy at $mGlu_5$, even at concentrations several orders of magnitude higher than its potency as a PAM (O'Brien et al., 2004; Chen et al., 2008). The same occurs with the $mGlu_1$ radioligand [^{3}H]R214127, which is thought to bind to site on $mGlu_1$ homologous with the MPEP binding site on $mGlu_5$ (Pagano et al., 2000; Lavreysen et al., 2003). CPPHA does not compete with [^{3}H]R214127 (Chen et al., 2008), despite having PAM activity at both group I mGlus. Furthermore, the neutral allosteric modulator 5MPEP noncompetitively inhibits CPPHA potentiation of glutamate, suggesting that ligands at the MPEP and CPPHA sites can allosterically regulate one another (Chen et al., 2008). This is in contrast to other $mGlu_5$ PAMs and NAMs that display a competitive interaction with 5MPEP (Chen et al., 2007). Collectively, these data suggest that CPPHA interacts with a different site to other known allosteric ligands. In site-directed mutagenesis studies, only F585I/$mGlu_5$, and its equivalent at $mGlu_1$ (F599I), in TM1 eliminated the potentiation of $mGlu_5$ by 1μM CPPHA (Chen et al., 2008). As seen in Fig. 4E, this residue on TM1 is spatially in a different region of the receptor compared to the other residues implicated in allosteric modulation in both $mGlu_1$ and $mGlu_5$. Because CPPHA potentiates responses to activation of both $mGlu_5$ and $mGlu_1$ in sites that are clearly distinct from previously characterized allosteric sites, the data suggests that multiple distinct allosteric sites exist on group I mGlus that can serve as targets for PAMs.

In a similarly designed study to the CPPHA work, two $mGlu_1$ selective PAMs, VU48 and VU71, were shown to be non-competitive with [^{3}H]R214127 and were not susceptible to mutations known to affect NAMs that bind the common group I mGlu allosteric site (Hemstapat et al., 2006). Multiple binding sites have also been suggested for $mGlu_4$ PAMs and $mGlu_2$ NAMs compared to PAMs. At $mGlu_2$, mutations known to perturb the allosteric modulation by PAMs had no effect on NAMs from the MNI series of compounds (Hemstapat et al., 2007). However, given that these $mGlu_2$ data rely upon functional measures alone, it remains to be seen whether or not these differential effects correspond to different binding sites or merely reflect the manifestation of positive versus negative cooperativity. Interestingly, at $mGlu_4$, PHCCC was unable to influence the concentration-response curve of VU0155041, a PAM from a different chemical class, suggesting that these two ligands do not act at a single allosteric site (Niswender et al., 2008). Furthermore, VU0155041 also has agonist activity in its own right, whilst PHCCC does not (Niswender et al., 2008), such differences in pharmacology of these PAMs could be postulated to arise due to interaction with different binding sites. It is possible that allosteric modulators acting at different sites could regulate mGlus via different pathways. Indeed, CPPHA has been shown to differentially effect ERK1/2 phosphorylation relative to inositol phosphate accumulation in rat cortical astrocytes (Zhang et al., 2005). In contrast, $mGlu_5$ PAMs from the CDPPB series have equivalent effects in both assays. Such differences between the activities of PAMs interacting at different sites have important implications with respect to therapeutic intervention and lead compound choice in drug discovery programs.

This evidence bears the potential that instead of (or in addition to) multiple well-defined and separated allosteric binding sites a larger binding region for allosteric modulation may exist defined by the inward-facing top half of the TM helices. Allosteric modulators interact with part of that region in multiple, possibly overlapping binding modes depending on their chemotype. Such a scenario increases the possibility to design allosteric modulators with finely tuned pharmacological effects as the determinants of the receptor/ligand interaction are optimized.

6. Conclusions

Since the discovery of the first mGlu allosteric modulator in 1996, the field of mGlu allosteric modulation has seen a number of major advances. For the majority of subtypes, selective allosteric modulators have now been discovered. Positive, negative and neutral allosteric ligands have been identified for some subtypes, providing much needed pharmacological tools to probe the physiological roles of these important receptors. With the clinical validity of allosteric modulation of GPCRs as a therapeutic avenue being proven, allosteric modulators of mGlus have considerable promise as candidates for therapeutic intervention for a variety of psychiatric and neurological disorders. Much progress has also been made in our understanding of the locations of allosteric binding sites on these receptors. The studies reported to date suggest both common binding sites across the different subtypes that can be selectively targeted as well as evidence for the presence of multiple binding sites on a single subtype. With the growing number of GPCR templates, information-rich homology models can be developed in the absence of a crystal structure for the transmembrane region of a class C GPCR. As presented above homology models are successfully utilized to interpret mutagenesis-based studies, allowing 3D visualization of these regions of the receptor.

Studies of mGlus have also utilized homology models to manually dock ligand-based pharmacophore maps to interpret structure activity relationships of a particular chemical series (Noeske et al., 2007, 2009). In the future, the combination of ligand-based and receptor-based pharmacophores (Kratochwil et al., 2005; Radestock et al., 2008) is likely to aid in the identification of novel chemotypes and optimization of allosteric modulators to improve specificity and potency for use as therapeutic agents.

Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.neuropharm.2010.07.007.

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